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Summary

Background: The success rate for vitrification of immature equine oocytes is low. Although vitrified-warmed oocytes are able to mature, further embryonic development appears to be compromised.

Objectives: The aim of this study was to compare two vitrification protocols, and to examine the effect of the number of layers of cumulus cells surrounding the oocyte during vitrification of immature equine oocytes.

Study design: Experimental *in vitro* and *in vivo* trials.

Methods: Immature equine oocytes were vitrified after a short exposure to high concentrations of cryoprotective agents (CPAs), or a long exposure to lower concentrations of CPAs. In Experiment 1, the maturation of oocytes surrounded by multiple layers of cumulus cells (CC oocytes) and oocytes surrounded by only corona radiata (CR oocytes) was investigated. In Experiment 2, spindle configuration was determined for CR oocytes vitrified using the two vitrification protocols. In Experiment 3, further embryonic development was studied after fertilization and culture. Embryo transfer was performed in a standard manner.
Results: Similar nuclear maturation rates were observed for CR oocytes vitrified using the long exposure and non-vitrified controls. Furthermore, a lower maturation rate was obtained for CC oocytes vitrified with the short exposure compared to control CR oocytes ($p = 0.001$). Both vitrification protocols resulted in significantly higher rates of aberrant spindle configuration than the control groups ($p<0.05$). Blastocyst development only occurred in CR oocytes vitrified using the short vitrification protocol, and even though blastocyst rates were significantly lower than in the control group ($p<0.001$), transfer of 5 embryos resulted in one healthy foal.
Main limitations: The relatively low number of equine oocytes and embryo transfer procedures performed.
Conclusions: For vitrification of immature equine oocytes, the use of (1) CR oocytes, (2) a high concentration of CPAs and (3) a short exposure time may be key factors for maintaining developmental competence.

Keywords: vitrification, cumulus cells, horse, oocyte

Introduction

Equine assisted reproductive technologies have evolved rapidly during the last decade and the relatively new techniques of cryopreserving immature oocytes may offer further advancement. For clinical application, it would allow postponement of the decision on the choice of stallion for intracytoplasmic sperm injection (ICSI). For research, it could provide a reliable source of immature equine oocytes in countries without access to equine slaughterhouses, such as the United States, assuming that such cells could be transported legally. Moreover, oocyte cryopreservation allows the preservation of genetics from valuable horses and endangered breeds [1]. However, the overall success rate of this technique in the horse is low. So far, there are only two studies reporting the birth of foals using vitrified oocytes that were partially matured *in vivo* [2; 3] while pregnancies obtained after fertilization of *in vitro* matured oocytes following vitrification-warming at the immature stage have not been reported yet. In a first study, Maclellan et al. [2] recovered oocytes by ovum pick-up 24-26 h after initiation of maturation *in vivo*, cultured in maturation medium for 2–4 h, vitrified-warmed, and then cultured for 10–12 h before subsequent transfer to inseminated mares for *in vivo* fertilization; this resulted in two live born foals. The same group also reported four pregnancies obtained from oocytes that were vitrified after initiation of maturation *in vivo*, fertilized by ICSI, *in vitro* cultured and transferred to recipient mares [3].

Vitrification is the most commonly used cryopreservation technique for oocytes. It is characterized by the use of high concentrations of cryoprotective agents (CPAs) and the fast cooling rate [4]. Oocytes from large domestic species are rich in cytoplasmic lipid droplets causing them to be highly sensitive to chilling [5], thus requiring species specific optimization of the exposure time and concentration of CPAs.

Successful vitrification is influenced by different factors that affect oocyte cryotolerance, including the presence of cumulus cells surrounding the oocyte at the time of vitrification. While a protective effect of cumulus cells during vitrification of immature oocytes has been reported [6-8], other studies show that cumulus cells constitute a tight multilayer barrier that reduces the entry of CPAs into the oocyte, thereby influencing the exchange of water and CPAs [9]. Removing all cumulus cells before vitrification of immature oocytes might result in a lower maturation rate and impaired embryo development [10]. Therefore, vitrification of oocytes that are surrounded only by corona radiata (CR)

cells has been proposed as a sound alternative in cattle [9], as the CR cells allow an appropriate exchange of water and CPAs, while the developmental capacity of the oocyte is maintained [9; 11].

The aim of the current study was to improve the vitrification protocol for immature equine oocytes. To this end, the effect of the number of cumulus cell layers (multiple layers of cumulus cells vs. corona radiata only) surrounding immature equine oocytes at the time of vitrification was evaluated, and two vitrification protocols were compared: one with a short exposure to a high CPA concentration, and one with a longer exposure to a lower CPA concentration.

Materials and methods

Media and reagents

Dulbecco´s Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12), Tissue Culture Medium-199 with Hanks' salts (TCM-199) and Hoechst 33342 were purchased from Life Technologies Europe. Unless otherwise stated, all other components were obtained from Sigma (Bornem, Belgium).

Maturation medium was composed of DMEM/F12 supplemented with serum replacement (9.4%), epidermal growth factor (0.05 µg/ml), follicle stimulating hormone (9.4 µg/ml), luteinizing hormone (1.88 µg/ml), glutamine (90 µg/ml), ascorbic acid (68 µg/ml), polyvinyl alcohol (23 µg/ml), myoinositol (4.5 µg/ml), Na pyruvate (99.5 µg/ml), insulin (9.5 µg/ml), transferrin (8.6 µg/ml), selenium (10 ng/ml), cysteine (0.094 mg/ml), cysteamine (0.046 mg/ml) and lactic acid (9.4 µl/ml).

Experimental design

In three consecutive experiments, immature equine oocytes were either vitrified with a short exposure to a high CPA concentration (further referred to as short vitrification protocol), or vitrified with a longer exposure to a lower CPA concentration (further referred to as long vitrification protocol), or not vitrified (control). In experiment 1, oocytes surrounded by multiple layers of cumulus cells (further referred to as CC oocytes, Figure 1A) or by corona radiata only (further referred to as CR oocytes, Figure 1B) were used. Fresh and vitrified-warmed CC and CR oocytes were matured *in vitro* and the nuclear maturation was evaluated. In Experiment 2, fresh and vitrified-warmed CR oocytes were matured *in vitro*, and meiotic spindle configuration was assessed. In Experiment 3, fresh and vitrified-warmed CR

oocytes were matured *in vitro*, and the developmental competence to the blastocyst stage was studied after fertilization by ICSI. Blastocysts obtained after vitrification with the short vitrification protocol were transferred on day 9 after ICSI to recipient mares.

Collection of equine immature oocytes

Equine ovaries were obtained from a local slaughterhouse, and transported in an insulated box to the laboratory at room temperature within 1 h. All follicles between 5 and 20 mm were aspirated using a 16-gauge needle attached to a vacuum pump (-100 mm Hg), scraped with the aspirating needle and flushed with TCM199 (Hanks).

Recovered oocytes were classified either as CC oocytes, which were surrounded by multiple layers of cumulus cells (Figure 1A) or as CR oocytes, which were surrounded by the corona radiata only (Figure 1B). Most of the recovered oocytes (more than 2/3) were classified as CC oocytes. To increase the number of CR oocytes, the excess cumulus cells in cumulus compact CC oocytes were removed by repeated pipetting of the oocyte inTCM199 (Hanks). As such, the CR oocytes used in this study were either directly collected from the slaughterhouse ovaries (less than 1/3) or obtained after repeated pipetting of cumulus compact CC oocytes. Due to our collection aspiration technique, we were unable to identify the directly collected CR oocytes as being either expanded or cumulus compact CC oocytes. Therefore, all the collected expanded oocytes were assigned to the CC oocytes in order to ensure that expanded CC oocytes were not over-represented in the CR group. Denuded and partially denuded oocytes were excluded from all experiments.

Recovered oocytes allocated to the control groups were immediately placed in maturation medium, while oocytes in the vitrified groups were first vitrified, and after one week of storage, they were warmed and incubated in maturation medium.

Vitrification and warming

The composition of the vitrification and warming solutions used in the two different protocols is given in Table 1. For both protocols, vitrification and warming steps were performed on a heated plate at 37°C. A custom-adapted device was used to store the oocytes in liquid nitrogen (LN_2). This device consisted of a 0.25 ml straw (IVM technologies, France) with a cut in one end to allow loading of the oocytes in a minimal volume (<1 µL). At the opposite end, a wire (obtained from a paper clip) was added to prevent

floating in LN_2 (Fig.1C–D). Oocytes were loaded using a 150 µm pipette in order to minimize the volume surrounding the oocytes. Moreover, extra medium surrounding the oocytes was removed with the pipette by capillarity.

Protocol with long exposure to low concentration of CPAs

This vitrification protocol was based on the one described by Kuwayama et al. [12] with some modifications, and will be referred to as 'long vitrification protocol'. Briefly, four oocytes at a time were placed into one single 75 µL droplet of handling solution (HS_L; long vitrification protocol), consisting of TCM199 (Hanks) supplemented with 20% (v/v) fetal bovine serum (FBS, Greiner Bio-one, Belgium). In order to allow a gradual equilibration of the oocytes, the HS_L droplet, containing the oocytes, was merged with a first 75 µL droplet of equilibration solution (ES) containing HS_L, with 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO). After 2 min, oocytes were transferred to the first ES droplet, which was merged with a second droplet of 75 µL containing ES, and oocytes were left there for 2 min. Next, oocytes were transferred to another droplet of 75 µL containing ES, and incubated for 6 min. Subsequently, oocytes were transferred into four consecutive 50 µl droplets of vitrification solution (VS), consisting of HS_L with 15% (v/v) EG, 15% (v/v) DMSO and 0.5 M sucrose for 60 s in total, and then loaded on a custom-adapted device (Figure 1C–D) and plunged into LN_2 within 10–20 s (Table 1).

After one week in LN_2, the custom-adapted device containing the four oocytes was introduced into 4 mL of warming solution (W1) containing HS_L supplemented with 1 M sucrose for 1 min. Next, oocytes were moved to 4 mL of W2 containing HS_L supplemented with 0.5 M sucrose for 3 min, and finally to 4 mL of W3 containing HS_L supplemented with 0.25 M sucrose for 5 min. Finally, oocytes were placed in 4 mL of HS_L, where they were stored until all oocytes were warmed (Table 1).

Protocol with short exposure to high concentration of CPAs

The second method of vitrification was based on the protocol described by Tharasanit et al. [13], with some modifications, and will be referred to as the 'short vitrification protocol'. Four oocytes at a time were placed in one single 100 µL droplet of handling solution (HS_S; short vitrification protocol) containing TCM199 (Hanks) supplemented with 0.014% (w/v) bovine serum albumin (BSA) for 1 min. The oocytes were then transferred to a 100 µL droplet of ES, consisting of HS_s supplemented with 10% (v/v) EG and

10% (v/v) DMSO. After 25 seconds, the oocytes were transferred to a 100 µL droplet of VS containing HS_s supplemented with 20% (v/v) EG, 20% (v/v) DMSO and 0.5 M sucrose. After 15 s, the oocytes were transferred to a custom-adapted device and plunged into LN_2 within 10–20 s.

After one week in LN_2, the custom-adapted device was transferred into 4 mL of W1 containing HS_s supplemented with 0.5 M sucrose, where oocytes were cultured for 5 min. Next, oocytes were stored in HS_s until warming of all oocytes was completed (Table 1).

In vitro maturation

A maximum of 40 oocytes at a time were transferred to 500 µL of DMEM/F12 based maturation medium [14] at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 28 h. For the exact composition of the maturation medium, we refer to the 'Reagents and Media' section. After maturation, oocytes were completely denuded by gentle pipetting in 0.05% (w/v) bovine hyaluronidase diluted in TCM199 (Hanks).

Evaluation of nuclear maturation (experiment 1)

For all six groups (CC and CR oocytes vitrified according to the long vitrification protocol, CC and CR oocytes vitrified according to the short vitrification protocol and fresh control CC and CR oocytes), maturation rates (see above) were determined by nuclear staining with 10 µg/ml Hoechst 33342. Oocytes were visualized and classified as metaphase I (MI, characterized by the presence of highly condensed chromosomes and the absence of the first polar body), metaphase II (MII, characterized by the presence of well-organized chromosomes and the presence of the first polar body) or degenerated (characterized by the absence of chromosomes) by epifluorescence microscopy using a Nikon TE300 inverted microscope with a 20× objective and equipped with a Nikon DS-Ri1 camera (Nikon Benelux, Zaventem, Belgium).

Spindle status assay (experiment 2)

Oocytes with a visible polar body after *in vitro* maturation (see above) were fixed in 4% (v/v) paraformaldehyde for 25 min, permeabilized with 0.5% (w/v) Triton X-100 for 1 h and blocked with PBS containing 10% (v/v) goat serum and 0.5% (w/v) BSA at 4ºC overnight. Subsequently, oocytes were incubated with anti-α-tubulin monoclonal antibody (Molecular Probes, Paisley, UK; 1:200 dilution)

overnight at 4°C followed by incubation with anti-mouse IgG antibody Alexa Fluor 488 (Molecular Probes; 1:500) for 1 h at 25°C. Oocytes were counterstained with 10 µg/ml Hoechst 33342 for 10 min and analysed using a Leica TCS-SP8 X confocal microscope (Leica Microsystems, Wetzlar, Germany). Chromosome and microtubule distributions were classified according to Tremoleda et al. [15]. Briefly, the meiotic spindle was defined as normal when it was symmetrically barrel-shaped with the two poles and two equal sets of chromosomes aligned at its centre (Figure 2A). On the other hand, abnormal spindles showed disorganized, clumped, dispersed or unidentifiable spindle elements (Figure 2B) with chromosome alignment defects (Figure 2C).

ICSI and embryo culture (experiment 3)

Oocytes showing an extruded polar body after *in vitro* maturation (see above) were fertilized by ICSI as described by Smits et al. [16]. Frozen-thawed semen from a single stallion of proven fertility was used, and spermatozoa were selected using 45%–90% Percoll (GE Healthcare, Belgium) density gradient centrifugation for 40 min at 750 x g at 26°C. After removal of the supernatant, the sperm pellet was washed in 5 ml of Ca^{2+}-free Sperm-TALP (tyrode´s albumin-lactate-pyruvate) using centrifugation for 10 min at 400 x g at 26°C. The supernatant was removed again and the sperm pellet was re-suspended in 300 µl of Ca^{2+}-free Sperm-TALP and kept at room temperature until used for ICSI. Immediately before ICSI, a small volume of sperm suspension was added to the left side of a 5 µl droplet of 9% polyvinylpyrrolidone in PBS and the spermatozoa were allowed to swim out to the right side of the droplet where they were picked up for ICSI.

All manipulations were performed on the heated stage (37°C) of an inverted microscope. A progressively motile spermatozoon was aspirated with its tail first into a blunt piezo pipet of 6 µm (Origio, Vreeland, The Netherlands) and immobilized by applying a few pulses of a piezo drill (Prime Tech, Ibaraki, Japan, speed: 4, intensity: 3) to its tail. Oocytes were held in separate 5 µL droplets of TCM199 (Hanks) containing 10% (v/v) FBS under mineral oil. The oocyte was fixed by aspiration with a holding pipet with an inner diameter of 15–20 µm (Origio, Vreeland, The Netherlands) keeping the polar body at 12 o'clock or 6 o'clock. The zona pellucida was drilled using the piezo (speed: 4, intensity: 3), a piece of zona was removed and after penetration of the oolemma with the piezo (speed: 3, intensity: 2), the spermatozoon was injected into the cytoplasm of the oocyte. Injected oocytes were cultured in groups of 10 to 20 in 20 µL droplets of DMEM/F12 with 10% (v/v) FBS at 38.2°C in a

humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Cleavage rate was determined on day 2.5 after fertilization and blastocyst development on day 9 post-fertilization.

Embryo transfer

The five blastocysts produced from vitrified-warmed oocytes were washed twice in preheated Emcare Holding Medium (ICPbio Reproduction, USA) and placed in a 2 mL tube filled with preheated Emcare Holding Medium. During transport to the embryo transfer centre (2 h), the tube was kept in 50 mL of preheated PBS in an insulated box. Upon arrival, the five blastocysts were washed again in Emcare Holding Medium and transferred transvaginally either singly (n = 3) or as a pair (n = 1) to the uterus of a recipient mare at day 4 or 5 post ovulation.

Data analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences (IBM® SPSS® Statistics 23.0, Chicago, IL). Two-way analysis of variance (ANOVA) was performed in order to evaluate the maturation rate outcome (MI and MII) and the proportion of degenerated oocytes (dependent variables). Fixed effects (independent variables) were classified according to the number of layers of cumulus cells (CC and CR), and the vitrification treatment (control, long vitrification protocol or short vitrification protocol), and their respective first degree interactions. Variables obtained from embryo development analysis, and spindle and chromosome alignment were evaluated using one-way ANOVA. The models included the likelihood of maturation, cleavage, blastocyst rate, and spindle and chromosome configuration as dependent variables. The treatment (control, long vitrification protocol and short vitrification protocol) was set as the categorical independent variable. For all outcome variables, the replicates were forced into the model to account for clustering of observations within a replicate. Results are expressed as mean ± standard deviation (s.d.) For all the models, statistical significance level was set at $p \leq 0.05$.

Results

Experiment 1: Effect of the number of layers of cumulus cells and vitrification protocol on the maturation rate of vitrified equine oocytes

Equine immature oocytes were used to evaluate the effect of the number of cumulus cell layers (CC vs. CR, Figure 1A–B) and the two vitrification protocols (long protocol vs. short protocol, Table 1) on the maturation rate of vitrified-warmed equine oocytes (Table 2). A clear association was observed between the number of cumulus cell layers and the vitrification treatment on maturation and degeneration rates.

The maturation rate of CR oocytes vitrified with the long vitrification protocol did not differ significantly from control oocytes. However, the maturation rate of CC oocytes vitrified with the short vitrification protocol (25.3%) was lower ($p = 0.01$) compared with control CC oocytes (53.4%), and with control CR oocytes (58.1%, $p = 0.001$). Also the maturation rate of CR oocytes vitrified with the short vitrification protocol (34.4%) was lower ($p = 0.03$) compared with control CR oocytes (58.1%).

Experiment 2: Effect of vitrification on spindle morphology of equine immature oocytes

We observed fewer oocytes with a normal spindle in vitrified oocytes compared with control oocytes (53.3%–68.4% vs. 81.3%, $p<0.05$; Figure 3). Furthermore, a lower percentage of a correct alignment of chromosomes was observed in vitrified oocytes compared with control oocytes (66.7%–68.4% vs. 87.5%, $p<0.05$, Figure 3). However, no significant differences were observed in the spindle morphology and chromosome alignment between vitrified groups (Figure 3).

Experiment 3: Effect of vitrification protocol on embryo development of vitrified equine immature oocytes

In a pilot experiment (n = 583), control and vitrified oocytes used in Experiment 1 were fertilized by ICSI and cultured for 9 d. Preliminary results showed that only CR oocytes vitrified with the short vitrification protocol had the potential to develop into a blastocyst (data not shown). According to the results from Experiment 1 and the pilot study, we decided to use only CR oocytes to investigate the effect of both vitrification protocols on embryo development (Table 3). Vitrification significantly decreased the number of cleaved oocytes in both methods ($p<0.05$). Furthermore, embryo development was significantly impaired after vitrification in both protocols ($p<0.05$), but only the oocytes vitrified with the short vitrification protocol showed potential to develop into a blastocyst (6.9%, $p = 0.001$).

All blastocysts obtained after vitrification of immature oocytes based on the short vitrification protocol were transferred on day 9 after ICSI to recipient mares at day 4 or 5 after ovulation. Initially, two blastocysts were transferred individually to two mares, but no pregnancies were observed. Next, two blastocysts were transferred to a single mare, and ultrasound revealed one embryonic vesicle, though no embryonic heartbeat could be detected at day 21 after transfer, so the pregnancy was lost. After transferring another blastocyst to a recipient mare, an embryonic vesicle was detected 9 days after transfer and a heartbeat was confirmed at 21 days after transfer. The pregnancy resulted in a healthy male foal, born on May 12th 2017 (Figure 4).

Discussion

In this study, we have demonstrated that immature CR equine oocytes can be vitrified successfully using a high concentration of CPAs and a short time of exposure to the equilibration and vitrification solutions. Vitrified oocytes generally showed lower maturation rates compared with controls, and the cleavage and blastocyst formation rates were significantly compromised. Nevertheless, we obtained an improved blastocyst development, and to the best of our knowledge, for the first time, a foal after transferring an *in vitro* produced (IVP) blastocyst, which was derived from an equine oocyte vitrified at the immature stage.

In the first experiment, we observed that the maturation of vitrified immature equine oocytes was strongly influenced by the number of cumulus cell layers surrounding the oocyte. In horses, a protective effect of cumulus cells during vitrification of immature oocytes has been suggested [6], but in that study, vitrification of cumulus-intact oocytes was compared with that of denuded oocytes, from which the cumulus cells had been removed completely. As the presence of cumulus cells is indispensable for maturation [10], the low maturation rate observed after vitrification of denuded oocytes might be a consequence of the total absence of cumulus cells, rather than the effect of the vitrification process. Therefore, CR oocytes were used in this study to evaluate the effect of the number of cumulus cell layers, as described previously in cattle [11]. We observed no significant difference in maturation rate between control fresh CC and CR oocytes, indicating that corona radiata cells have the capacity to support the oocyte reaching MII as adequately as intact CC oocytes. In our study, the presence of multiple layers of cumulus cells surrounding the oocyte combined with the use

of a short vitrification protocol, i.e. less than one min., negatively affected survival and further maturation. We presumed that this effect was related to the layers of cumulus cells surrounding the oocyte, and not to the potential difference between CC and CR oocytes, because we took steps to ensure that cumulus oocytes were not over-represented in the CR oocyte group. Cumulus cells may impair the movement of CPAs into the oocyte leading potentially to an inappropriate intracellular CPA concentration, as the efflux of water from oocytes occurs quickly, within 20 s, whereas the influx of CPAs takes longer [17].

Exposing oocytes to CPAs during vitrification induces osmotic volume changes due to the migration of water and CPAs. In mature oocytes, these volume changes might cause a disruption of the spindle while in immature oocytes, microfilament organization might be disturbed [18; 19]. In our study, vitrification resulted in significantly higher percentages of oocytes with abnormal spindle structures associated with disorganized microtubules when compared to control oocytes. No significant differences were observed amongst the vitrified groups, even though oocytes vitrified with the short vitrification protocol were exposed to more extreme osmotic changes as a consequence of the higher concentration of CPAs used.

Vitrification of immature oocytes is generally associated with a significant decrease in blastocyst development in mammalian species [7; 13; 20], because cryopreservation can induce a rupture of the oolemma [21; 22] and cytoskeletal disorganization [23]. In our study, cleavage and blastocyst rates were indeed severely reduced in vitrified oocytes when compared to control oocytes. Although oocytes vitrified with both protocols did not show a significantly different cleavage rate, blastocyst development was only observed in oocytes vitrified with the short vitrification protocol. Almost 7% of the injected oocytes developed into a blastocyst, i.e. 16% of the cleaved embryos.

The short vitrification protocol used in this study, was previously described by Tharasanit et al. [13]. These authors reported 1% blastocyst formation after vitrification of immature equine oocytes. The higher blastocysts rate obtained in this study (7%) may be the result of the two modifications included. Besides the fact that we used CR oocytes, a custom adapted device similar to the cryotop was used as an alternative to the open pulled straw (OPS) that was used by Tharasanit et al. [13]. Using this device, oocytes are loaded in a minimum volume of vitrification solution (<1 µl), resulting in faster

cooling-warming rates than with the OPS, a modification that was demonstrated to lead to higher cleavage and blastocyst rates in other species [24-26].

Surprisingly, we observed that maturation rates in CR oocytes vitrified with the long vitrification protocol did not appear to be strongly reduced (no significant difference with control fresh oocytes), but they were not able to develop into blastocysts. Canesin et al. [27] have recently published development of one blastocyst out of nine oocytes using this long protocol for vitrification of equine oocytes. However, these authors used a different device to load the oocytes and different concentrations of sucrose during warming, which may explain in part, the disparate results.

More interestingly, Canesin et al. [27] reported that the concentration and time of exposure (more than 10 min) to CPAs used in the long vitrification protocol were detrimental for the developmental competence of immature equine oocytes. Indeed, CPA toxicity is considered as the most limiting factor when developing a successful vitrification protocol. The toxicity of penetrating CPAs consistently increases with higher CPA concentrations, higher exposure temperature, and/or longer exposure time [28]. Although higher concentrations of CPAs were used in the equilibration and vitrification solutions of the short vitrification protocol, the very strongly reduced time of exposure could have resulted in the successful cryopreservation, as previously reported in bovine oocytes [29].

The ultimate goal of oocyte vitrification is to preserve developmental capacity to the blastocyst stage, resulting in a successful pregnancy and a live foal. Live births have been reported after vitrifying immature bovine [30] and porcine [20] oocytes; however, as far as we know, no pregnancies or foals born from vitrified immature oocytes after complete IVP have been reported. In the current study, we report a successful equine pregnancy resulting in a healthy foal (Figure 4), which is a major achievement in the field of equine assisted reproduction, and equine oocyte cryopreservation in particular.

In conclusion, we developed an improved method for the vitrification of immature equine oocytes. Although the blastocyst rate was compromised, blastocyst development using our vitrification protocol was enhanced and did result in a healthy foal. Nevertheless, further research is needed to reduce the ultrastructural spindle alterations observed in vitrified oocytes and, concurrently, to improve normal resumption of meiosis and subsequent blastocyst development.

Author's declaration of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical animal research

Oocyte experiments: Research ethics committee oversight not currently required by this journal: the study was performed on material obtained from an abattoir. Embyro transfer: Prof Dr K. Hermans, Chairperson Ethical Committee, Faculty of Veterinary Medicine, University of Ghent has confirmed that ethical committee oversight is not required for this component of the study in the institute where the work was performed.

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Authorship

N. Ortiz-Escribano and K. Smits conceived the idea and experimental design was developed together with H. Woelders, C. De Schauwer, J. Govaere, E. Van den Abbeel, C. Ververs, K. Roels, M. Van De Velde and A. Van Soom. N. Ortiz-Escribano and K. Smits performed the experiments with the support of O. Bogado Pascottini, L. Vandenberghe, T. Vullers, C. Ververs, K. Roels, M. Van De Velde and H. Woelders, C. De Schauwer, J. Govaere, E. Van den Abbeel and A. Van Soom contributed to data interpretation. H. Woelders, E. Van den Abbeel and A. Van Soom supervised the project and N. Ortiz-Escribano, K. Smits and A. Van Soom wrote the manuscript with input from O. Bogado Pascottini, H. Woelders, L. Vandenberghe, C. De Schauwer, J. Govaere and E. Van den Abbeel. All authors have approved the final version of the manuscript.

Manufacturers' addresses

[a]Life Technologies Europe.
[b]Sigma, Bornem, Belgium.
[c]IVM Technologies, France.
[d]Greiner Bio-one, Belgium.
[e]Nikon Benelux, Zaventem, Belgium.
[f]Molecular Probes, Paisley, UK.
[g]Leica Microsystems, Wetzlar, Germany.
[h]GE Healthcare, Belgium.
[i]Origio, Vreeland, The Netherlands.
[j]Prime Tech, Ibaraki, Japan.
[k]ICPBIO Reproduction, USA.
[l]SPSS®, Chicago, Illinois, USA.

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Table1. Composition of vitrification-warming solutions and time of exposure used in the different protocols.

	Long vitrification protocol		Short vitrification protocol	
	Solution	Time	Solution	Time
Handling solution (HS)	TCM199Hanks + 20%FBS	1'	TCM199Hanks + 0.014%BSA	1'
Equilibration solution (ES)	HS+ 7.5%EG + 7.5%DMSO	10'	HS + 10%EG + 10%DMSO	25´´
Vitrification solution (VS)	HS+15%EG +15%DMSO + 0.5M sucrose	1'	HS+20%EG+20%DMSO+0.5M sucrose	15''
Warming (W1)	HS + 1M sucrose	1'	HS + 0.5 M sucrose	5'
Warming (W2)	HS + 0.5M sucrose	3'	-	
Warming (W3)	HS + 0.25M sucrose	5'	-	

FBS: Fetal Bovine serum, EG: Ethylene Glycol, DMSO: Dimethyl sulfoxide

Table 2. Overview of the *in vitro* maturation rates of control and vitrified immature equine oocytes surrounded by multiple layers of cumulus cells (CC) and by corona radiata cells only (CR) using two different vitrification protocols.

Treatment	Group	n	Undefined	MI (%)		MII (%)	Degenerated (%)
Control	CC	88	13	12(13.6±7.1)	⎤	47(53.4±19.4)^{ab}	16(18.2±6.8)^{ab}
	CR	86	16	9(10.5±3.7)	⎦	50(58.1±7.8)^{a}	11(12.8±4.3)^{a}
Long vitrification	CC	93	9	20(21±6.8)	⎤	32(34.4±9.5)^{bc}	32(34.4±15)^{ab}
	CR	122	13	16(13.1±5.2)	⎦	59(48.4±12.6)^{ab}	34(27.9±17)^{ab}
Short vitrification	CC	99	12	21(21.2±6.5)	⎤*	25(25.3±8.3)^{c}	41(41.4±12.5)^{b}
	CR	122	20	25(20.5±12.4)	⎦	42(34.4±5)^{bc}	35(28.7±11.4)^{ab}

For MI, main effects were analyzed, and differences were only observed between the treatments. * The control group was significantly different from the group vitrified with the short vitrification protocol ($p<0.05$).^{a,b,c} Groups with different superscripts within the same column are significantly different ($p<0.05$). Data are given as mean percentages ± s.d. (five replicates, n = 610).

Table 3. Overview of the maturation rate and embryo development of control and vitrified-warmed equine immature oocytes surrounded only by corona radiata cells, using two vitrification protocols (long vitrification protocol vs. short vitrification protocol).

Group	n	MII oocytes (%)	Cleavage (%)	Blastocyst (% of injected oocytes)	Blastocyst (% of cleaved zygotes)
Control	146	80 (54.8±7.3)^{a}	61 (76.3±8.4)^{a}	16 (20±2)^{a}	16 (26.2±1.7)^{a}
Long vitrification protocol	141	56 (39.7±9.9)^{b}	17 (30.3±7)^{b}	0^{b}	0^{b}
Short vitrification protocol	179	72 (40.2±3.9)^{b}	30 (41.6±19.9)^{b}	5 (6.9±5.9)^{c}	5 (16.7±17.3)^{a}

^{a,b,c} Groups with different superscripts within the same column are significantly different ($p<0.05$). Data are given as mean percentages ± s.d. (four replicates, n = 465).

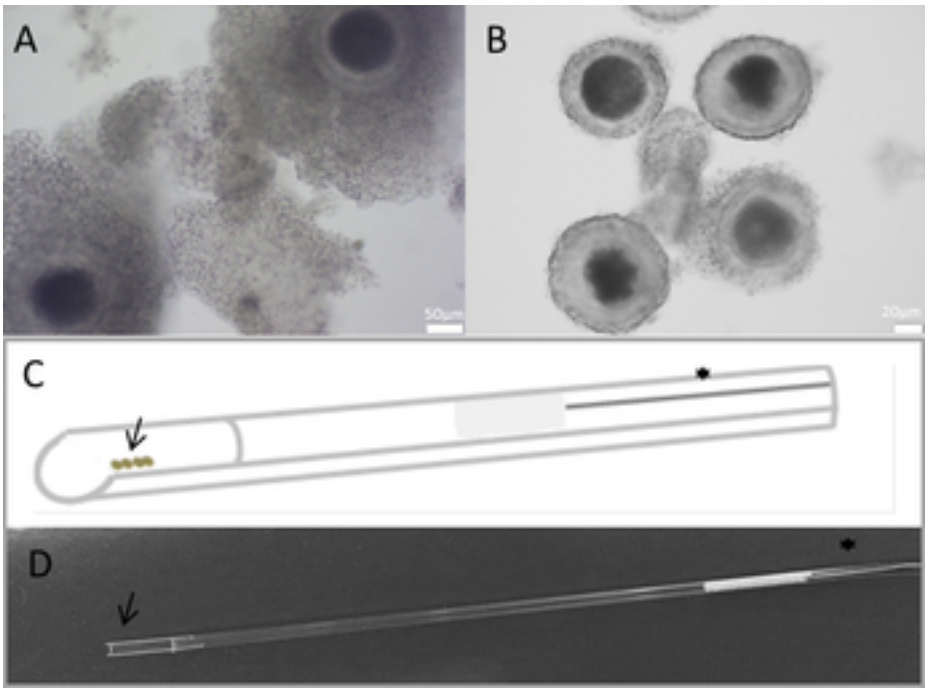

Figure 1. (A–B) Representative images of oocytes surrounded by multiple layers of cumulus cells (A) and oocytes surrounded by corona radiata cells only (B). (C–D) Drawing and representative images of the custom-adapted device used in the present study. The arrows denote where the oocytes are loaded and the asterisks denote the wire added.

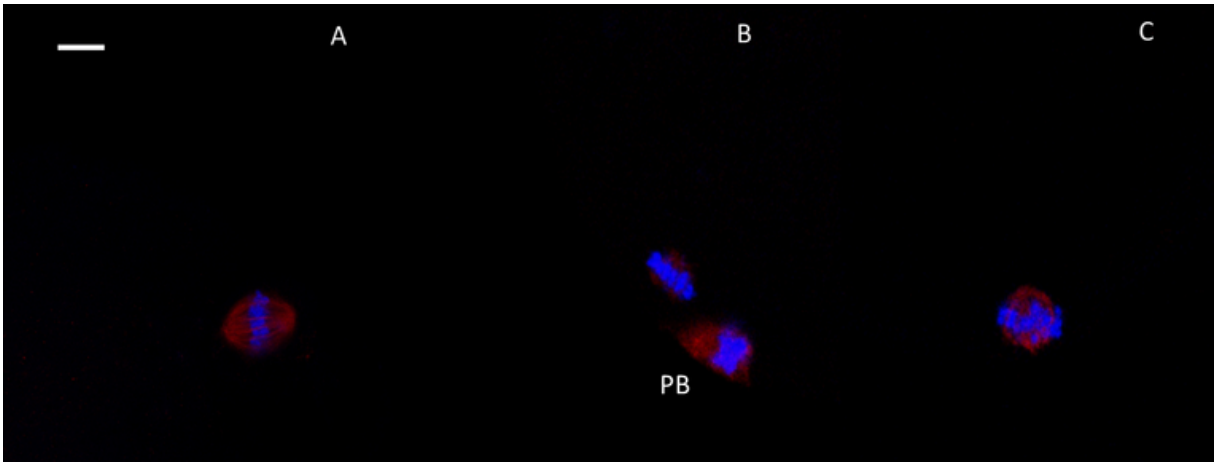

Figure 2. (A–C) Confocal images illustrating cytoskeleton morphology in vitrified-warmed oocytes. Microtubules are stained in red and chromatin in blue. (A) shows a normal MII oocyte with its typical barrel-shaped metaphase II spindle configuration and the chromosomes perfectly aligned in blue. (B) shows an oocyte with a extruded polar body (PB), smaller spindle and aligned chromosomes.(C) shows oocyte with a disrupted spindle and dispersed chromosomes.

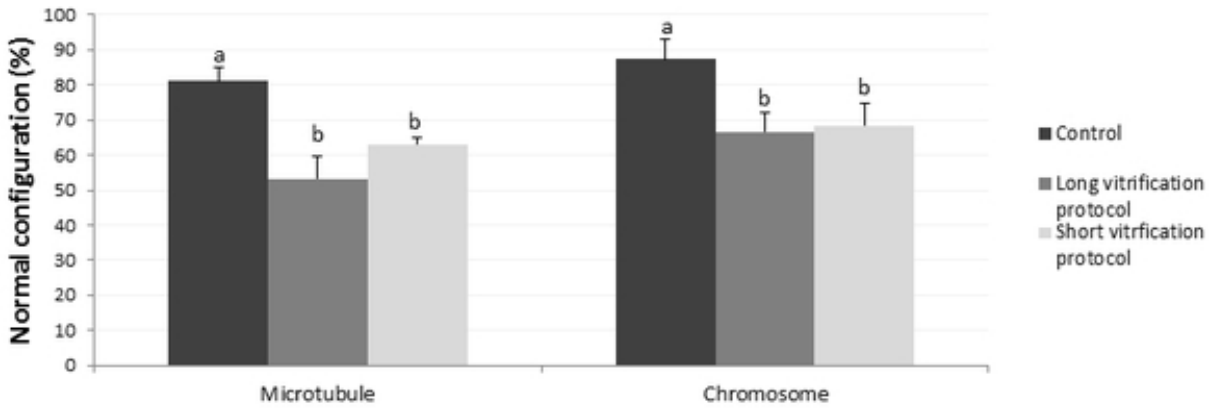

Figure 3. Percentage of normal microtubule and chromosome alignment of control and vitrified oocytes. [a,b,] Groups with different superscripts are significantly different ($p<0.05$). Three replicates, n = 50.

Figure 4. A healthy male foal was born at term, here depicted at 3 days of age.